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Generation of MHC class I-restricted cytotoxic T lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells

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SUMMARY

Expression of reporter genes in muscle cells has been achieved by intramuscular (i.m.) injection of plasmid DNA expression vectors. We previously demonstrated that this technique is an effective means of immunization to elicit both antibodies capable of conferring homologous protection and cell-mediated immunity leading to cross-strain protection against influenza virus challenge in mice. These results suggested that expression of viral proteins by muscle cells can result in the generation of cellular immune responses, including cytotoxic T lymphocytes (CTL). However, because DNA has the potential to be internalized and expressed by other cell types, we sought to determine whether or not induction of CTL required synthesis of antigen in non-muscle cells and, if not, whether transfer of antigen to antigen-presenting cells from muscle cells may be involved. In the present study, we demonstrate that transplantation of nucleoprotein (NP)-transfected myoblasts into syngeneic mice led to the generation of NP-specific antibodies and CTL, and cross-strain protective immunity against a lethal challenge with influenza virus. Furthermore, transplantation of NP-expressing myoblasts (H-2^k) intraperitoneally into F1 hybrid mice (H-$2^d \times$ H-2^k) elicited NP CTL restricted by the MHC haplotype of both parental strains. These results indicate that NP expression by muscle cells after transplantation was sufficient to generate protective cell-mediated immunity, and that induction of the CTL response was mediated, at least in part, by transfer of antigen from the transplanted muscle cells to a host cell.

INTRODUCTION

Direct injection of plasmid DNA encoding reporter genes has been shown to result in protein expression *in situ*.[1] Many different genes have been expressed in a variety of species, including rats, rabbits, dogs, fish, chickens, ferrets, cattle and non-human primates. Intramuscular (i.m.) injection of plasmid DNA provides a simple means of obtaining sufficient expression of proteins *in vivo* for generating effective humoral and cellular immune responses. Antibodies and/or cytotoxic T lymphocytes (CTL) have been generated in animals injected with DNA encoding viral proteins such as influenza virus nucleoprotein (NP) and hemagglutinin, human immunodeficiency virus (HIV) envelope proteins, bovine herpesvirus gIV, hepatitis B surface antigen, rabies virus glycoprotein, and hepatitis C virus nucleocapsid; parasite proteins such as the circumspozoite protein of malaria; bacterial proteins such as *Leishmania major* gp63, and *Mycobacterium tuberculosis* antigen 85 and heat-shock protein (hsp 65); and other proteins such as carcinoembryonic antigen, major histocompatibility complex (MHC) molecules, and antibodies (for review see[2]). In some instances, these immune responses sufficed to provide protection against subsequent challenge with live pathogen. For example, the efficacy of vaccination with DNA was first demonstrated using a plasmid encoding a conserved internal protein of influenza virus, nucleoprotein (NP), and resulted in protective cell-mediated immunity in mice against challenge with a strain of influenza A virus very different from the strain used to clone the NP gene.[3] Protection in animal models was subsequently demonstrated by injection of DNA encoding antigens from malaria, bovine herpesvirus, rabies virus, papilloma virus, herpes simplex virus, *Mycoplasma*, and lymphocytic choriomeningitis virus (for review see [2]).

The mechanisms of DNA uptake by cells *in vivo* and presentation of expressed antigen to the immune system after DNA injection are not yet known. Preliminary evidence suggests that muscle cells may play a role in these processes. First, protein expression was observed in muscle cells after intramuscular (i.m.) administration of DNA in saline.[1] Expression was also seen after direct DNA injection into a variety of tissues and organs,[1] or in dermal and epidermal cells after gene gun inoculation.[4] However, if no mechanical force was used to propel DNA directly into cells, the level of expression was highest by far in muscle.[1] Second, although CTL responses could be induced by intradermal and intravenous administration of NP DNA, significant cell-mediated immune protection from cross-strain influenza virus challenge

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was only conferred by i.m. injection.[2] Thus, there may be a physiological process of DNA uptake present in muscle cells. It is not yet known why muscle cells are more capable of internalizing plasmid DNA and/or expressing the gene than other types of cells, but injury to the large, multinucleated myocytes does not appear to be a factor.[5,6] Morphologic studies have implicated cell membrane invaginations, termed caveolae, and T-tubules, which are preponderant in myocytes, in DNA uptake.[7] These results, taken together, suggested that synthesis of antigens by muscle cells is effective for the generation of MHC class I-restricted immune responses after i.m. injection of DNA. However, muscle cells are not considered to be antigen presenting cells and it is possible that non-muscle cells could internalize injected plasmid DNA after i.m. injection. For example, circulating cells present in the muscle at the time of injection or shortly thereafter, or those cells to which DNA may have been carried by the circulation might internalize some of the injected DNA and serve as antigen presenting cells. However, surveys of non-muscle tissues for the presence of plasmid DNA following i.m. injection have revealed the presence of little or no plasmid in non-muscle tissues, as judged by a sensitive polymerase chain reaction (PCR) technique.[8] Furthermore, the *in vitro* half-life of naked plasmid DNA in serum is < 30 min (unpublished observations) and the *in vivo* half-life of DNA formulated with cationic lipids is < 5 min.[9] It has not yet been determined whether transfection of non-muscle cells by i.m.-injected DNA, if it occurs, plays a role in the generation of immune responses seen after DNA vaccination. In addition, it is not known whether proteins or fragments thereof synthesized by transfected muscle cells are transferred to host cells for induction of CTL. Therefore, the purposes of these studies were to investigate the sufficiency of antigen synthesis by muscle cells to produce protective immune responses against a viral protein and to investigate the participation of non-muscle cells in this process. Here we demonstrate that antigen synthesis by muscle cells alone is sufficient for the induction of protective cell-mediated immunity (CMI), and that transfer of antigen expressed by muscle cells to a host antigen presenting cell can occur.

MATERIALS AND METHODS

Mice

C3H/HeN mice, C3D2 (C3H × DBA/2) F1 hybrid mice, and CC3 (BALB/c × C3H) F1 hybrid mice (The Jackson Laboratory Bar Harbor, ME), and BALB/c mice (Charles River Laboratories Raleigh, NC) were used for transplantation and/or primary myoblast explants.

Cell lines

C_2C_{12} (H-2K^k) cells were obtained from ATCC (Rockville, MD) and maintained in high glucose DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone Labs, Inc., Logan, UT), penicillin, streptomycin, and glutamine.

Flow cytometry

Two days prior to staining for MHC class I expression, C_2C_{12} myoblasts were cultured with or without 100 U/ml recombinant mouse γ-interferon (Genzyme, Cambridge, MA). Myoblasts were removed from culture flasks using trypsin–ethylene diamine tetraacetic acid (EDTA) (Gibco). Cells were washed once with phosphate-buffered saline (PBS) containing 5% FBS (Gibco) and sodium azide, counted and 1×10^6 cells were aliquoted into each of three microfuge tubes to serve as unstained, anti-H-2K^k stained or isotype control stained samples. Cells were pelleted by centrifugation at 15 600 *g* for 10 seconds. Unstained cells were resuspended in 50 μl of PBS/FBS. Cells to be stained for MHC class I expression were resuspended in 40 μl of PBS/FBS and 10 μl fluorescein isothiocyanate (FITC)-conjugated mouse H-2K^k antibody, immunoglobulinG-1 (IgG1) isotype, (200 μg/ml) (PharMingen, San Diego, CA). Isotype controls were stained with an unrelated monoclonal antibody (PharMingen). All samples were placed on ice for 1 hr, protected from light. Cells were then washed twice with 1 ml of PBS/FBS, resuspended in 1 ml of Haema-line 2 (Serono Baker Diagnostics, Allentown, PA), acquired (5000 events) and analysed on a Becton Dickinson fluorescence activated cell sorter (FACScan) (Becton Dickinson Immunocytometry Systems, Braintree, MA).

Primary myoblast explants

Primary neonatal myoblast cultures were prepared and maintained as described by Tautu *et al.*[10] with the following modifications. Quadriceps muscles were removed 2 to 4 days after birth and the tissue was dissociated by exposing the muscle to two 30 min incubations with cold Hanks balanced salt solution containing 0·5% trypsin, with occasional gentle agitation. After triturating with a 25 ml pipette to disaggregate particulate material, the suspension was centrifuged at 433 *g*, 4° for 10 min. Cells were then resuspended in modified Eagle's medium (MEM) supplemented with 10% horse serum (Gibco), 1·5% chick embryo extract (Gibco) buffered with HEPES, followed by filtration through gauze and subsequently 200, 100, 48, and 20 μm mesh. After the cultures reached ~60% confluency, the media were replaced with fresh media, gently swirled across the growth surface to remove loose cells, decanted and finally replaced with fresh media. When approximately 75% confluent, cells were split by trypsinization and resuspended at 1×10^5 cells/ml for further growth in culture. Cells were always maintained below confluency.

Enzyme-linked immunosorbent assay (ELISA) for anti-NP antibody

Polyvinyl chloride ELISA plates (Costar, Cambridge, MA) were coated with 100 μl/well of NP (purified from insect cells that had been transfected with a baculovirus expression vector)[3] at 10 μg/ml in PBS and incubated overnight at 4°. Plates were washed three times with PBS + 0·05% Tween (wash buffer) and blocked with PBS + 0·05% Tween + 0·1% bovine serum albumin (BSA) (blocking buffer) at 22° for 1 hr. Serum samples were added in tenfold serial dilutions in blocking buffer, and incubated for 1 hr at 22° with gentle agitation. The plates were then washed three times as before and incubated with 100 μl/well of horseradish peroxidase-conjugated goat anti-mouse IgGF_c (Jackson ImmunoResearch, West Grove, PA), (1:2000 dilution, 22° for 1 hr). Substrate development was carried out using *o*-phenylenediamine (Sigma, St. Louis, MO) (1 mg/ml in 0·1 M citric acid buffer, pH 4·5 + 0·012% peroxide), and absorption was measured at 450 nm (A^{450}). The same procedure was used for determination

of immunoglobulin isotypes, except that conjugated isoytpe-specific secondary antibodies (Zymed) were used.

CTL assays

CTL assays were performed as previously described[3] with the following modifications. Spleen cells were restimulated with recombinant human interleukin-2 (IL-2) plus syngeneic spleen cells that had been either infected with influenza A virus (A/PR/8/34) or pulsed with NP peptides; H-2^k (amino acids 50–57; SDYEGRLI) and H-2^d (amino acids 147–155; TYQRTRALV). CTL effector activity was determined using the following cell types as targets. For H-2^k, C_2C_{12} cells, either undifferentiated or differentiated, or L929 cells were used (peptide-pulsed at 10 μg/ml, or infected with influenza virus A/Victoria/73, or NP-transfected). For H-2^d, P815 cells were used as above. All target cells were plated two days prior to assay with or without the addition of 100 U/ml interferon-γ. The appropriate cells were virally infected while attached in tissue culture flasks for 1 hr at 37° followed by two washes of the monolayer with 3 ml PBS + 0·1% BSA and the addition of 1 ml of RPMI-1640 medium supplemented with 10% FBS, penicillin, streptomycin, HEPES and glutamine. After labeling with 200 μCi ^{51}Cr, cells were washed once, incubated for 10 min with fresh media, and washed again. Adherent cells were removed from culture flasks by the addition of 1 ml trypsin–EDTA for 1 min, washed and resuspended in 1 ml media, counted and plated with effectors. Suspension cells were washed by sedimentation and resuspension. CTL assays were performed for 3 hr as before.[3]

Transfection of myoblasts

C_2C_{12} myoblasts were transfected with V1J-NP plasmid DNA[11] either with or without SV2 neo (ATCC) using a calcium phosphate transfection procedure as outlined in the CellPhect Transfection Kit (Pharmacia, Piscataway, NJ). Transient transfectants were prepared for injection 24 hr after transfection with V1J-NP. Stable transfectants were selected by incubation in geneticin (Sigma) at 1·2 mg/ml. Several clones were selected by limiting dilution and further selected for high-level NP expression as measured by immunoblot, immunofluorescence staining and CTL-mediated recognition and lysis. The level of NP expression was estimated by immunoblot analysis using known amounts of recombinant NP as standard.

Influenza virus challenge

A/HK/68 influenza virus was diluted in DMEM (high glucose) supplemented with 0·1% BSA, penicillin, streptomycin and glutamine. Mice were anesthetized with an i.p. injection (0·3–0·4 ml) of a ketamine (Miles, Inc., Shawnee Mission, KA)/xylazine solution (Aveco Co., Inc., Fort Dodge, IA) (6·4 and 0·4 mg/ml respectively), and $10^{2.5}$ tissue culture infective dose 50% ($TCID_{50}$) of virus was administered intranasally. Weight loss and survival were monitored over a 21-day period. To generate a source of influenza-specific CTL for CTL assays, unanesthetized mice were infected with A/PR/8/34 influenza virus (10^3 $TCID_{50}$) by intranasal instillation.

RESULTS

Preparation of NP-expressing myoblasts

C_2C_{12} myoblasts were stably co-transfected with separate plasmids containing the neomycin resistance and NP genes. Several clones were isolated on the basis of antibiotic resistance and NP expression, as measured by immunoblot and FACscan analysis. These stable transfectants were further characterized with respect to recognition and lysis by CTL. The NP-transfected myoblasts were readily lysed by influenza-specific CTL, as were normal, virus-infected myoblast targets (Fig. 1). Target cells were either untreated or pre-incubated with interferon-γ (IFN-γ) for 2 days, which has been shown to upregulate MHC class I molecules.[12] By FACscan analysis, the level of MHC class I molecules on the myoblasts was substantially increased by this treatment (data not shown). Both untreated and IFN-γ treated transfectants were recognized and lysed by influenza-specific CTL. These results indicate that the transfected myoblasts expressed NP, proteolytically processed NP for antigen presentation and presented antigenic peptides in association with MHC class I for recognition by NP-specific CTL, even in the absence of IFN-induced upregulation of MHC class I molecules. Myoblasts stably transfected with the NP gene were used for subsequent transplantation studies.

Humoral immune responses generated by antigen expression in muscle cells *in vivo*

To obtain expression of a foreign protein exclusively in muscle cells *in vivo*, NP-transfected myoblasts were transplanted into naive, syngeneic mice (C3H/HeN) by i.m. injection into the quadriceps muscles. By PCR analysis, persistence of myoblasts and/or fusion with myocytes were demonstrated by the presence of NP DNA in DNA extracts of muscle necropsied up to at least 41 days after transplantation (data not shown). The steady-state level of NP expression in muscles was below the limit of detection by immunoblot analysis (< 1 ng, as estimated using purified, recombinant NP). However, NP expression *in vivo* after DNA injection was demonstrated directly by immunofluorescence staining of muscle tissue

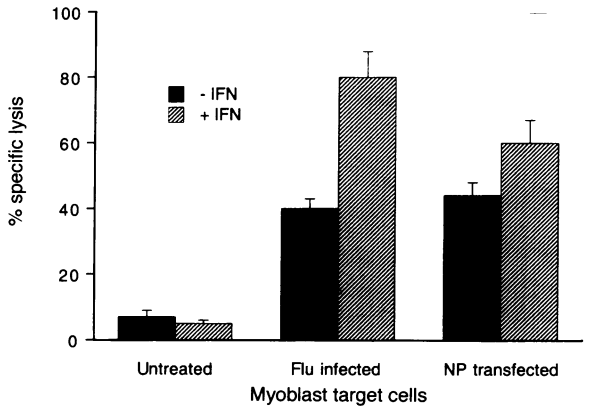

Figure 1. Transfection of myoblasts with NP DNA and recognition by CTL *in vitro*. C_2C_{12} myoblasts were stably transfected with NP DNA, as described in Materials and Methods. Normal and NP-transfected myoblasts were preincubated without (solid bars) or with (striped bars) IFN-γ (100 U/ml) for 2 days. As a positive control, normal myoblasts were infected with influenza virus A/Victoria/73. Target cells were loaded with ^{51}Cr and incubated with influenza virus-specific CTL for 3 hr (E:T = 25:1). Data are presented as percent specific lysis ± SD, where n = 3.

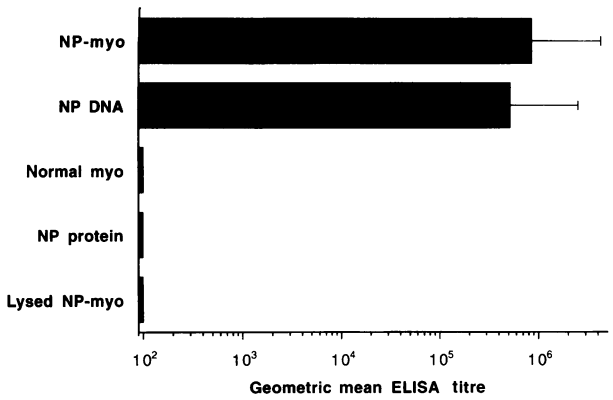

Figure 2. Generation of anti-NP antibodies by transplantation of transfected myoblasts. NP-transfected C_2C_{12} myoblasts (NP-myo) (1×10^7 cells) were transplanted into C3H/HeN mice by i.m. injection into the quadriceps muscles. As a positive control, mice were injected with NP DNA (100 μg/quadriceps). As negative controls, mice were either injected with non-transfected C_2C_{12} myoblasts (normal myo), an amount of recombinant NP protein equivalent to about five times that present in the transplanted myoblasts (0·1 μg/quadriceps), and NP-transfected myoblasts (1×10^7 cells) lysed by repeated freezing/thawing (lysed NP-myo). The presence of circulating anti-NP antibodies was measured by an ELISA four weeks after treatment. Data is presented as geometric mean ELISA endpoint titre ± SD, where $n = 10$.

sections (M. Cartwright *et al.*, unpublished observations) and indirectly by the generation of anti-NP antibodies (Fig. 2). Anti-NP antibody titres after transplantation (endpoint titres of $\sim 10^6$) were comparable to those generated by i.m. injection of NP DNA. Higher anti-NP antibody titres were consistently observed in mice that received transfected myoblasts at multiple sites in the quadriceps muscles, compared to those who received the same total number of cells in a single site. This humoral immune response after transplantation was likely not due to NP

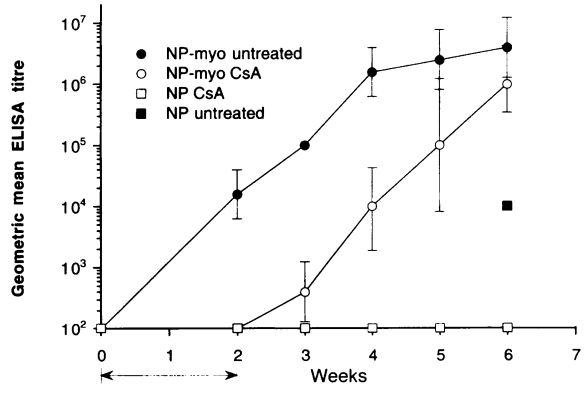

Figure 3. Effect of immunosuppression on kinetics of appearance of anti-NP antibodies after transplantation. C3H/HeN mice were injected with NP-transfected myoblasts (open and solid circles) or NP protein (1 μg) (open and solid squares). Some of the mice were injected daily with cyclosporin A (50 μg/g) (open symbols) or untreated (solid symbols) for two weeks (arrow). Serum was collected at weekly intervals and anti-NP antibodies were assayed by an ELISA. Mice injected with 1 μg NP protein and no cyclosproin A treatment had endpoint titres of $\sim 10^4$ by six weeks (solid square). Data are presented as geometric mean ELISA endpoint titre ± SD, where $n = 5$.

Table 1. Immunoglobulin isotypes of anti-NP antibodies

Inoculum	IgG1	IgG2a	IgG2b	IgG3
NP-myoblasts	19 905	> 500 000	79 244	< 500
NP DNA	7924	> 500 000	79 244	< 500
Live virus	2811	88 914	< 500	1581
NP protein	315 480	315 480	31 547	< 500
Normal myoblasts	< 500	< 500	< 500	< 500

Geometric mean ELISA endpoint titre for anti-NP antibodies from serum samples taken 5 weeks after inoculation, where $n = 10$.

release from lysed myoblasts shortly after transplantation, since no anti-NP antibodies were generated in mice injected with non-viable transfectants or mice injected with an amount of purified NP protein (0·1 μg) equivalent to about five times that present in the injected transfectants. Moreover, transplanted mice that were immunosuppressed by daily injections of cyclosporin A (CsA) beginning the day of transplantation developed anti-NP antibodies upon discontinuation of CsA treatment (Fig. 3). The ensuing kinetics of antibody appearance and magnitude of titre were similar to those in untreated transplanted mice. In contrast, mice immunized with 1 μg of NP protein generated anti-NP antibodies by 6 weeks, while similarly immunized mice treated with CsA for 2 weeks did not. Therefore, the appearance of anti-NP antibodies after removal of the CsA-induced immunosuppression indicates that the expression of NP occurred in persistent myoblasts and/or mature myocytes as a result of fusion with transfected myoblasts. Based on these results and the observation that NP DNA is present in muscle 18 weeks after i.m. injection,[8] NP expression may persist in muscle cells after DNA vaccination.

The antibody responses induced by expression in muscle cells after transplantation were predominantly of the IgG2a isotype, with lesser levels of IgG2b and IgG1, and little IgG3 (Table 1). This isotype profile was similar to that induced by NP DNA vaccination and live virus infection, but different from that after inoculation with recombinant NP protein. The latter generated substantially higher levels of IgG1 (~10- to 100-fold). Anti-NP antibody responses could be induced by transplantation of as few as 10^4 transfected myoblasts (data not shown).

Cell-mediated immune responses induced by antigen expression in muscle cells *in vivo*

As previously reported, i.m. injection of NP DNA resulted in the generation of NP-specific CTL, in addition to NP antibodies.[3] Therefore, we were interested to determine whether expression of NP in muscle cells after transplantation was sufficient to elicit NP CTL. Spleen cells were isolated from mice transplanted with transfected myoblasts, and CTL were restimulated *in vitro* with influenza virus-infected syngeneic spleen cells from naive mice. Transplantation of NP-transfected myoblasts into naive mice generated NP-specific CTL; levels of ^{51}Cr-release were similar in magnitude to those generated by spleen cells from influenza virus-infected and NP DNA-injected mice (Fig. 4). The target cells used were either virus-infected or pulsed with the H-2^k MHC class I-restricted peptide. Untreated C_2C_{12}

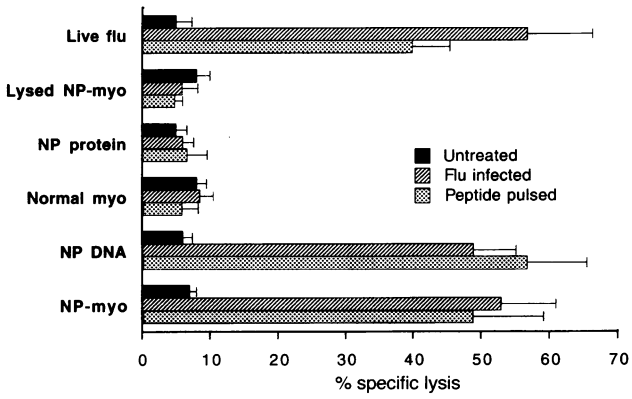

Figure 4. Generation of NP-specific CTL after transplantation of transfected C_2C_{12} myoblasts. C3H/HeN mice were injected with NP-transfected myoblasts (NP-myo) (1×10^7 cells), NP DNA (100 μg), normal C_2C_{12} myoblasts (normal myo) (1×10^7 cells), or NP protein (1 μg/quadriceps). CTL were prepared from these mice 6 weeks after treatment. As a negative control, CTL were prepared from mice that received NP-transfected myoblasts (1×10^7 cells) lysed by repeated freezing/thawing (lysed NP-myo). As a positive control, CTL were prepared from mice that had been infected with influenza virus A/HK/68. Untreated (stippled bars), influenza virus A/Victoria/73-infected (solid bars) and NP-transfected myoblasts (striped bars) were used as target cells. CTL were incubated with ^{51}Cr-labelled targets for 3 hr (E:T = 25:1). Data are presented as percent specific lysis ± SD, where $n = 3$.

targets were not lysed by the CTL. Similar results were obtained using MHC haplotype-matched mouse L929 fibroblasts as targets (data not shown). Therefore, the CTL generated in mice by transplantation of NP-expressing myoblasts were directed toward an NP epitope and not that of another protein expressed by the myoblasts. NP-specific CTL were not generated in mice injected with NP protein or non-viable transfectants. NP-specific CTL were also induced in naive mice that were transplanted with syngeneic primary myoblasts expressing NP following transient transfection with NP DNA (data not shown). Both virus-infected and peptide-pulsed L929 target cells were recognized and lysed. Mock-transfected primary myoblasts did not induce NP-specific CTL. Therefore, expression of NP in muscle cells *in vivo* was sufficient to induce NP-specific CTL.

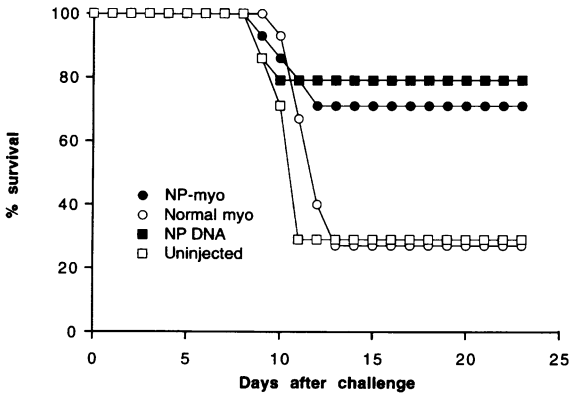

Figure 5. Generation of protective immunity by expression of NP in muscle cells. C3H/HeN mice were injected with NP DNA (100 μg/quadriceps) (solid squares), NP-transfected C_2C_{12} myoblasts (1×10^7 cells) (solid circles), normal myoblasts (1×10^7 cells) (open circles) or were uninjected (open squares). Mice received three injections at 3-week intervals. Three weeks after the final injection, mice were challenged with a lethal dose of influenza virus A/HK/68 ($10^{2.5}$ $TCID_{50}$). Data are presented as percent survival in groups of 15 mice.

Protective immunity induced by antigen expression in muscle cells *in vivo*

Injection of NP DNA into mice conferred cell-mediated, protective immunity against a cross-strain challenge with influenza A virus.[3,13] Furthermore, $CD8^+$ T cells were shown to play a key role in this protection (unpublished observations). To determine whether NP expression by muscle cells after transplantation would be sufficient to confer protective immunity, transplanted mice were challenged with a lethal dose of influenza A virus. The challenge strain of virus used was A/HK/68, a different subtype that arose 34 years after the A/PR/8/34 strain from which the NP gene was cloned. Mice that received either NP DNA or NP-transfected myoblasts

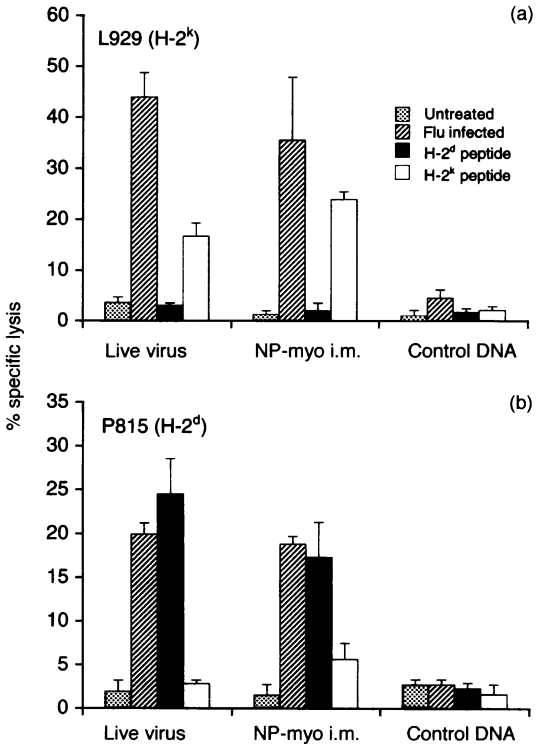

Figure 6. Induction of $H\text{-}2^k$ and $H\text{-}2^d$ in CC3 F1 hybrid mice. CC3 mice were injected i.m. with NP-expressing myoblasts (10^7 cells), infected with influenza virus (A/PR/8/34) (10^3 $TCID_{50}$), or were injected with control DNA. Spleen cells were restimulated in culture with irradiated syngeneic F1 spleen cells infected with influenza virus and tested against L929 ($H\text{-}2^k$) or P815 ($H\text{-}2^d$) target cells at an effector:target ratio of 25:1 against untreated (stippled bars), influenza virus-infected (A/Victoria/73) (striped bars), $H\text{-}2^d$ peptide-pulsed (solid bars) or $H\text{-}2^k$ peptide-pulsed (open bars) cells. Data are presented as percent specific lysis ± SD, where $n = 3$. Note the difference in magnitude of the y-axes of the two panels.

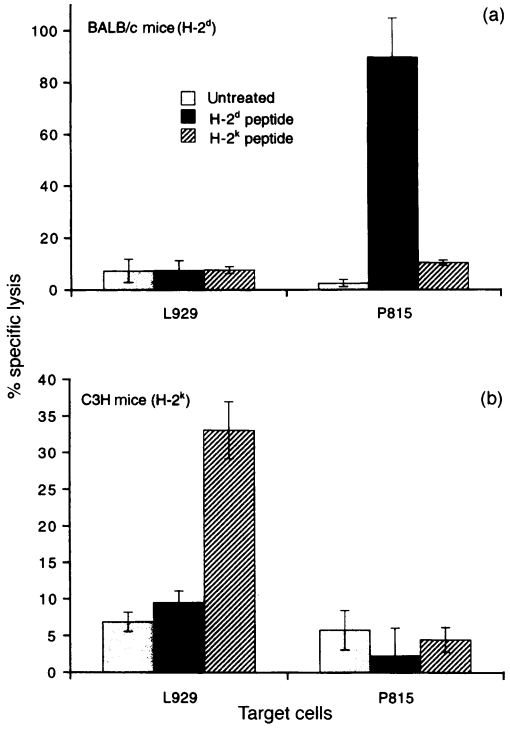

Figure 7. Specificity of H-2^d- and H-2^k-restricted NP CTL epitopes. BALB/c (a) and C3H (b) mice were infected with influenza virus (A/PR/8/34) and spleen cells were restimulated with syngeneic spleen cells infected with influenza virus. CTL were assayed at an effector:target ratio of 50:1 against L929 cells (H-2^k) and P815 cells (H-2^d) that were untreated (stippled bars), H-2^d peptide-pulsed (solid bars), and H-2^k peptide-pulsed (striped bars). Data are presented as percent specific lysis ± SD, where $n = 3$.

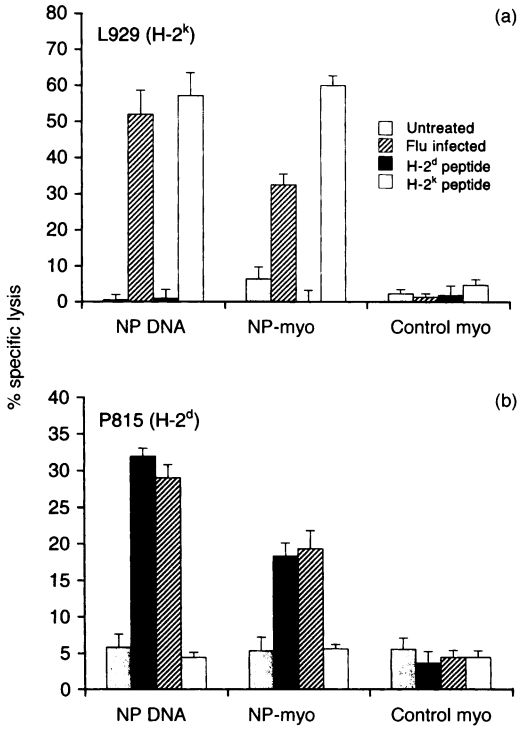

Figure 8. Generation of H-2^d CTL after transplantation of H-2^k myoblasts into CC3 F1 hybrid mice (H-2^d × H-2^k). CC3 mice were given NP-expressing myoblasts (H-2^k) or normal, untransfected myoblasts (10^7 cells) by i.p. injection or NP DNA by i.m. injection. Spleen cells were restimulated in culture with irradiated syngeneic F1 spleen cells infected with influenza virus and tested against L929 (H-2^k) (a) or P815 (H-2^d) (b) target cells at an effector:target ratio of 50:1. Target cells were untreated (stippled bars), influenza virus-infected (A/Victoria/73) (striped bars), H-2^d peptide-pulsed (solid bars) or H-2^k peptide-pulsed (open bars). Data are presented as percent specific lysis ± SD, where $n = 3$. Note the difference in magnitude of the y-axes of the two panels.

showed greater survival (79 and 71%, respectively), compared to mice that were uninjected or transplanted with normal myoblasts (29 and 27%, respectively) (Fig. 5). In this particular experiment, mice were either injected or transplanted 3 times at 3 week intervals and challenged 3 weeks after the final dose. In a similar experiment, a single transplantation of NP-transfected myoblasts followed by challenge after 9 weeks also resulted in greater survival over controls (~50% net increase; data not shown). Therefore, NP expression by muscle cells *in vivo* is sufficient to generate protective immunity against a lethal challenge with influenza virus.

Induction of H-2^d-restricted CTL after transplantation of myoblasts (H-2^k) into F1 hybrid mice (H-2^d × H-2^k)

To investigate the involvement of non-muscle cells in the induction of CTL responses after transplantation of NP-expressing myoblasts, F1 hybrid mice were used in which one of the parental strains was of the same MHC haplotype as that of the myoblasts (H-2^k). In this way, the myoblasts should not be rejected by the host due to histocompatibility differences and, by determining the MHC restriction of the CTL responses induced in the F1 hybrid mice, one can assess whether or not host cells play a role in the presentation of antigen to the immune system. The F1 hybrid mice were capable of generating CTL responses to distinct NP peptides presented by the two corresponding parental MHC haplotypes (H-2^k and H-2^d), as demonstrated with spleen cells from influenza virus-infected mice and mice injected i.m. with NP-expressing myoblasts or NP DNA (Fig. 6; see also Fig. 8). The H-2^d CTL responses appear to be inherently lower than the H-2^k in this F1 hybrid mouse, as the percentage of specific lysis by H-2^d CTL induced by various means of immunization was consistently lower (observed in 9 of 10 experiments). The H-2^k and H-2^d NP peptides are not cross-reactive, as evidenced by a lack of lysis of target cells of the inappropriate haplotype restriction (Fig. 7). Expression of NP in myocytes either as a result of DNA uptake after DNA injection or myoblast fusion after myoblast transplantation could result in presentation of NP epitopes by both MHC haplotyes present in the host myocytes. Hence, to delineate between antigen presentation by the muscle cells or a non-muscle host cell, NP-expressing myoblasts were transplanted intraperitoneally (i.p.); a site where fusion with skeletal muscle cells or other host cells would not be expected to occur. In this way, expression of NP would be limited to the transplanted myoblasts. Transfer of DNA from the transfected myoblasts to other cells leading to NP expression in a non-muscle cell is highly unlikely because the transfected cells

used contained a stably integrated NP gene(s). Intraperitoneal (i.p.) transplantation of NP-expressing myoblasts into sygeneic H-2^k mice induced CTL of similar magnitude to that after influenza virus infection or NP DNA injection (data not shown). In CC3 (BALB/c × C3H) F1 hybrid mice, i.p. transplantation of NP-expressing myoblasts resulted in the generation of CTL that recognized distinct peptides presented by both H-2^k and H-2^d MHC haplotypes (Fig. 8). Therefore, because CTL responses were induced that recognized a peptide presented by an MHC class I haplotype not present on the transplanted myoblasts (H-2^d), antigen or processed peptide most likely was transferred from these muscle cells to a host cell for presentation to the immune system.

DISCUSSION

Direct injection of plasmid DNA encoding an antigen into skeletal muscle results in protein expression in myocytes and in some cases induction of CTL leading to protective immunity, suggesting that muscle cells may play a role in these processes.[2] It is possible, however, that non-muscle cells could also internalize DNA and express NP after i.m. injection of DNA, and thereby serve as antigen presenting cells. This could include antigen-presenting cells within the muscle body or at other sites to which DNA has been carried by the circulation. To address the issue of antigen presentation after DNA vaccination, we used the technique of myoblast transplantation to express the viral protein NP in muscle cells *in vivo* (for example see [14]). The data presented here demonstrate that synthesis of NP by muscle cells alone is sufficient to generate protective immunity, but that non-muscle cells can subsequently present this antigen to the immune system.

NP CTL and protection have been reported in mice after administration of 10 μg of NP protein.[15] In our hands, however, injection of recombinant NP protein (3 × 5 μg) did not induce CTL or protection.[16] Whether these disparate results are due to differences in the physical nature of the NP preparations is not known (e.g. the presence of other influenza proteins or particulate NP may facilitate processing for presentation by MHC class I). However, the amounts of NP administered in those studies is several orders of magnitude higher than the amount of NP expressed by the transfected myoblasts in our studies (~20 ng over a 3 day period). Therefore, the transfer of antigen to antigen-presenting cells observed after transplantation may not involve simple uptake of exogenous antigen following release from muscle cells. For example, it may require antigen to be in a different form than that produced in cells (e.g. as a peptide or as a complex with other cellular components) or be present in a different location in the body than the muscle. Alternatively, specialized antigen presenting cells capable of internalizing and presenting exogenous NP may not be present in the muscle after injection of NP protein, but could be attracted to the muscle in response to expression of NP by myocytes or injection of DNA itself. With respect to the latter, recent studies have shown that specific oligonucleotide sequences contained within certain types and forms of bacterial DNA are stimulatory for lymphocytes.[17] Yet, whatever the mechanism, uptake of DNA and synthesis of antigen by non-muscle cells is not necessary to account for the CTL responses seen after DNA injection.

Both NP-specific IgG antibodies and CTL were generated after transplantation of NP-transfected myoblasts. The production of anti-NP antibodies indicates that NP was either expressed on the surface of the myocytes or released by the cells. NP does not contain an amino-terminal signal sequence that would target it for secretion or residence on the plasma membrane, but release of NP was observed in transfected cells *in vitro* which could not be accounted for by cell lysis (unpublished observations). In addition, cell surface expression of NP has been seen in transfected and influenza virus-infected cells.[18] Anti-NP antibodies induced by a DNA vaccine would not be predicted to be neutralizing and, as expected, did not contribute to the observed protective immunity, since passive transfer of NP antibodies into naive mice did not decrease the levels of influenza virus in the lungs after challenge.[3] In addition to antibody and CTL generation, the release of NP by myocytes *in vivo* could also result in processing and presentation of NP in an MHC class II-restricted fashion by bone marrow-derived antigen-presenting cells. Indeed, lymphocyte proliferative responses were seen after antigen restimulation of spleen cells from NP DNA-injected mice (unpublished observations). These responses may be important for the generation of the observed T-dependent antibodies and CTL.

The production of NP-specific CTL by expression of NP in muscle cells, after either DNA injection or myoblast transplantation, indicates that NP was processed and presented to the immune system in association with MHC class I molecules. However, the nature of the antigen presenting cell(s) involved is currently not known. Possible ways in which DNA vaccines could induce CTL include: (i) antigen presentation by the muscle cells themselves, (ii) direct transfection of professional antigen presenting cells, or (iii) transfer of antigen from transfected muscle cells to professional antigen presenting cells. With respect to the first possibility, the role of muscle cells in immunological reactions has been controversial. Some studies have demonstrated that myoblasts can induce tolerance *in vivo*,[19,20] possibly due to the lack of costimulatory molecules, while others have shown that myoblasts can act as antigen presenting cells *in vitro* for the stimulation of $CD4^+$ T cells, following induction by IFN-γ and tumour necrosis factor-α (TNF-α).[21] Myoblasts and myocytes express MHC class I molecules, and can be recognized and lysed by CTL *in vitro*[12] (see also Fig. 1). Whether CTL-mediated lysis of muscle cells occurs *in vivo* after injection of DNA is not yet known but, based on the proportion of myocytes transfected after injection of reporter gene constructs (< 1%),[1] this would not affect many cells. The levels of expression of the costimulatory molecules necessary for effective stimulation of CTL, such as B7-1 and B7-2, in myocytes *in vivo* is not known. Myocytes do, however, produce an IL-2-like cytokine, termed IL-15,[22] which may bypass the requirement for costimulation by signalling through the γ_c chain of the IL-2 receptor.[23] Other non-professional antigen-presenting cells (i.e. fibroblasts) were recently shown to efficiently induce CTL *in vivo* and this depended upon MHC class I expression by the fibroblasts.[24] However, this response required that the fibroblasts be in the context of a lymphoid organ. In addition, induction of robust CTL is thought to be enhanced by cytokines secreted by helper T cells, as a consequence of presentation of antigen by MHC class II molecules. Muscle cells can be induced to express MHC class II

in vitro,[21] but *in vivo* expression has only been demonstrated in patients with neuromuscular diseases.[25] Therefore, based on current understanding, it seems unlikely that presentation of antigens for MHC class I-restricted responses expressed after DNA injection is mediated directly by myocytes. The second possibility involves transfection of professional antigen-presenting cells. This could theoretically occur after i.m. injection of DNA, but our data indicate that this is not necessary for induction of CTL and, if it occurs, does so at a very low level: (i) NP expression by muscle cells alone is sufficient to generate NP CTL after transplantation of myoblasts (transfer of genetic material from these stably transfected cells leading to expression in a host cell is very unlikely), and (ii) little or no plasmid DNA sequences were detected in any tissue other than the injected muscle after i.m. injection of plasmid.[8] Furthermore, a murine retroviral vector given i.m. induced CTL against an encoded protein and was similarly localized to the injection site.[26] The third possibility, namely that transfected muscle cells may serve as a source of newly synthesized antigen with subsequent transfer to a professional antigen-presenting cell, offers the best explanation. This would account for the observed induction of H-2^d-restricted CTL after transplantation of H-2^k myoblasts into F1 hybrid mice (H-2^d × H-2^k) and could explain how CTL are generated after transfection of muscle cells by i.m. injection of DNA. In support of this hypothesis, the transfer of tumour-specific antigen to bone marrow-derived antigen-presenting cells has been shown to play a role in the induction of CTL after implantation of tumour cells in mice.[27] In addition, coinjection of DNA constructs encoding rabies virus glycoprotein and granulocyte–macrophage colony stimulating factor (GM-CSF) enhanced the immune responses against the viral antigen,[28] which could be attributed to the stimulatory effects of GM-CSF on differentiation of professional antigen presenting cells.

Since NP appears to be released by the myocytes after DNA injection or transplantation, one potential way in which NP could be presented by MHC class I molecules on non-muscle cells is by a recently proposed mechanism involving the internalization of exogenous antigens.[29] Dendritic cells and macrophages have the capability of processing certain exogenous proteins for presentation by MHC class I and could be responsible for the presentation of NP leading to the generation of memory T cells. Because expression of antigen in muscle cells alone is sufficient to generate protective cell-mediated immunity, the possibility of specifically targeting DNA for uptake by muscle cells is attractive. The limitation of DNA uptake to muscle cells would be potentially advantageous from both safety and immunological considerations. First, because myocytes are terminally differentiated and non-dividing, the likelihood of integration of plasmid DNA is substantially lower than in a dividing cell.[30] Second, protein expression by muscle cells after DNA injection can in some instances persist for periods up to 19 months in mice[31] which may be important for maintaining long-lived, robust immune responses.[32] So far, NP DNA has been detected in muscle by PCR for up to at least 18 weeks after DNA injection.[8]

In summary, expression of proteins in muscle cells *in vivo* after myoblast transplantation can be used as a means of providing non-muscle proteins for the induction of immune responses, including antibodies and CTL. While it is still possible that muscle cells themselves act as antigen presenting cells, our data suggest that transfer of antigen from transfected muscle cells to MHC class I molecules of host antigen presenting cells can occur, and that antigen presenting cells of the host may contribute significantly to the induction of CTL responses induced by DNA vaccines.

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